

MODULE 7- LECTURE 1

MICROBIAL BIOTECHNOLOGY: GENETIC MANIPULATION

7-1.1 Introduction

Microbial biotechnology involves the exploitation, genetic manipulation and alterations of micro-organisms to make commercial valuable products and that also involves fermentation and various upstream and downstream processes.

Microorganisms produce an amazing array of valuable products such as macromolecules (e.g. proteins, nucleic acids, carbohydrate polymers, even cells) or smaller molecules and are usually divided into metabolites that are essential for vegetative growth (primary metabolites) and those which give advantages over adverse environment (secondary metabolites). They usually produce these compounds in small amounts that are needed for their own benefit.

7-1.2 Genetic Engineering of Microorganisms for Biotechnology

Molecular genetics can be used to manipulate genes in order to alter the expression and production of microbial products, including the expression of novel recombinant proteins.

The compounds that are isolated from plants or animals can be synthesized by genetic manipulation of different micro-organisms to enhance the production and by environmental and other manipulations, even up to 1000-fold for small metabolites can be increased.

The advent of recombinant DNA technology (also referred to as gene cloning or *in vitro genetic manipulation*) has dramatically broadened the spectrum of microbial genetic manipulations. With the advancement of recombinant DNA technology, many novel host systems have been explored to produce commercially important products like therapeutic proteins, antibiotics, small molecules, biosimilars etc.

The basis of this technology is the use of restriction endonucleases, polymerases and DNA ligases as a means to specifically cut and paste fragments of DNA. Similarly, foreign DNA fragments can be introduced into a vector molecule (a plasmid or a bacteriophage), which enables the DNA to replicate after introduction into a bacterial cell.

The ability to modify and clone genes accelerated the rate of discovery and the development in biotech industries.

The basic steps in DNA cloning involves the following,

- A fragment of DNA is inserted into a carrier DNA molecule, called a vector, to produce a recombinant DNA.
- The recombinant DNA is then introduced into a host cell, where it can multiply and produce numerous copies of itself within the host. The most commonly used host is the bacteria, although other hosts can also be used to propagate the recombinant DNA.
- Further amplification of the recombinant DNA is achieved when the host cell divides, carrying the recombinant DNA in their progenies, where further vector replication can occur.
- After a large number of divisions and replications, a colony or clone of identical host cell is produced, carrying one or more copies of the recombinant DNA.
- The colony carrying the recombinant DNA of interest is then identified, isolated, analyzed sub-cultured and maintained as a recombinant strain.

7-1.3 Expression of a foreign protein in a microbe

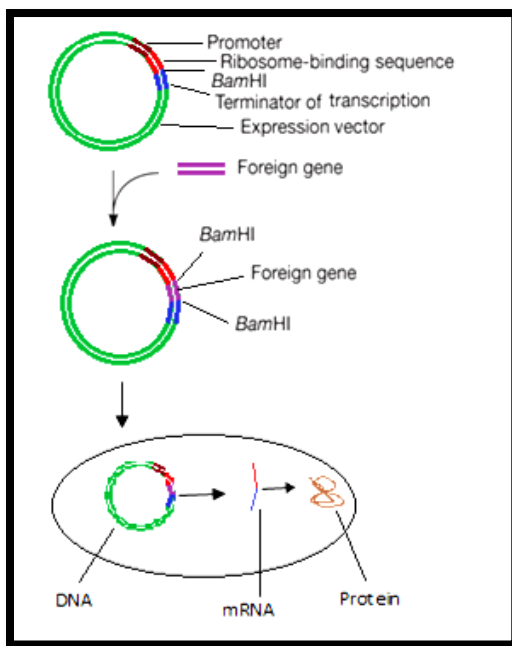


Figure7-1.3. Foreign protein expression in a microbe

There are several methods by which genetic alterations of producer microbial strains can be done for maximization of products or metabolites.

7-1.3.1 Traditional Method of Strain Improvement

- The remarkable increases in antibiotic productivity and the resulting decreases in costs have resulted due to mutation and screening for higher producing microbial strains. In recent years, efforts have been devoted to miniaturize and automate the screening procedures and to enhance the frequency of improved strains by selection procedures, e.g., the isolation of anti-metabolite-resistant mutants in cases where the natural metabolite is a precursor, an inhibitor or a co-repressor of a biosynthetic pathway.
- Mutation has also served to shift the proportion of metabolites production in a fermentation broth to a more favorable distribution, to elucidate the pathways of secondary metabolism, and to yield new antibiotics.
- Targeted mutagenesis: It involves introduction of mutations at a specific location in DNA. As many antibiotics, growth hormones, regulatory factors production genes have now been cloned, targeted mutagenesis of the cloned DNA can be performed *in vitro*, followed by transformation of the recipient organism.

- The major problems of the classic strain improvement procedure based on random mutagenesis were the very low probability of introducing mutations into relevant genes and high rate of unwanted mutations in other, unrelated genes.

7-1.3.2 Classical Genetics

The most effective use of classical genetics in the past was the backcrossing of overproducing strains with parent strains to improve the vigor of mutant strains.

- After backcrossing such a strain with the wild type, progeny cells are produced that have inherited the overproducing traits from the mutant parent and the wild-type hardiness and vigor from the wild-type parent.
- Priorly this was not possible with *Penicillium*, which lacks a true sexual cycle. However, a parasexual cycle resulting in the production of heterokaryons was discovered in *Penicillium* in 1958 and was used to improve the strains.
- Protoplast fusion is relatively a new versatile technique to induce or promote genetic recombination in a variety of prokaryotic and eukaryotic cell especially, industrially useful microorganisms such as *Streptomyces ambofaciens*, *Micromonospora chinosporea*, because it breaks the barriers to genetic exchange. Another use of protoplast fusion has been the recombination of different strains from the same or different species to yield new antibiotics such as anthracyclines, aminoglycosides and rifamycins. Protoplast fusion has also been useful in elimination of an undesirable component from penicillin broths imposed by conventional mating systems.

7-1.3.3 Rational Selection

It involves selecting an improved producer out of a very large population of progeny. For example, some antibiotics, notably penicillin and tetracyclines, are chelators of heavy metal ions. The more of these antibiotics an organism produces, the more resistant it will be to heavy metals in the medium. Thus, selection for mutants resistant to heavy metals was used in the improvement of the penicillin producers.

7-1.3.4 Cloning of the candidate genes

Recombinant DNA technology can be used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into non-producing strains to obtain modified or hybrid antibiotics. The use of recombinant DNA technology in antibiotic improvement and discovery has been enhanced by the finding that some streptomycetes antibiotic biosynthetic pathways are coded by plasmid genes, eg. methylenomycin A. Even when the antibiotic biosynthetic pathway genes of streptomycetes are chromosomal, they appear to be clustered into operons which facilitate transfer of an entire pathway in a single manipulation.

The genes encoding individual enzymes of antibiotic biosynthesis which have already been cloned include those of the cephalosporin, clavulanic acid, prodigiosin, undecylprodigiosin, actinomycin, and candicidin pathways. The isopenicillin N synthetase ("CyClase") gene of *Cephalosporium acremonium* has been cloned in *Escherichia coli* and expressed at a level of 20% of total cell protein. Cyclase gene of *Penicillium chrysogenum* and *Streptomyces clavuligerus* has also been cloned in *E.coli* system. The expandase/ hydroxylase gene of *C. acremonium* has been cloned in *E. coli*. The protein accumulated as inclusion bodies in *E. coli* near to 15% of total cell protein

Similar to *E.coli*, now a days *Bacillus subtilis*, *Pichia pastoris*, *Saccharomyces cerevisiae* have also emerged as a promising heterologous expression system for prokaryotic and eukaryotic candidate genes.

There are several factors which govern the production of recombinant therapeutic proteins in

B. subtilis. The factors are as follows:

- a) Well understood transcription and translation machinery including different regulatory factors responsible for extracellular product.
- b) Generation of stable recombinant plasmids.
- c) Development of novel recombinant *B. subtilis* strains with reduced nuclease and protease contents.
- d) Better systematic understanding of the protein secretion method to elucidate the factors responsible for the secretion of intracellular proteins.

To get the soluble extracellular protein product from the gene, it can be linked to a DNA fragment coding for *B. subtilis* signal peptide for extra-cellular secretion. The signal sequence may be preceded by a efficient translation initiator sequence or ribosome binding site (RBS) which is followed by mRNA stability-enhancing sequences (SES) at 5' end of mRNA. The 'strong' promoter for this gene consist a cluster of other efficient promoters which would be regulated temporally by the growth condition or growth medium components. Thus genes possess suitable properties not only for efficient transcription, translation but would be under temporal control avoiding other exogenous induction. Thus generated mRNA would be stabilized by 3'SES which protects mRNA from degradation by 3' exonucleases. The protein product consists of a typical *B. subtilis* signal peptide linked to N-terminus and have to be further processed with proteases to recover only the desired portion.

7-1.3.5. Microbial Cell-Surface Display

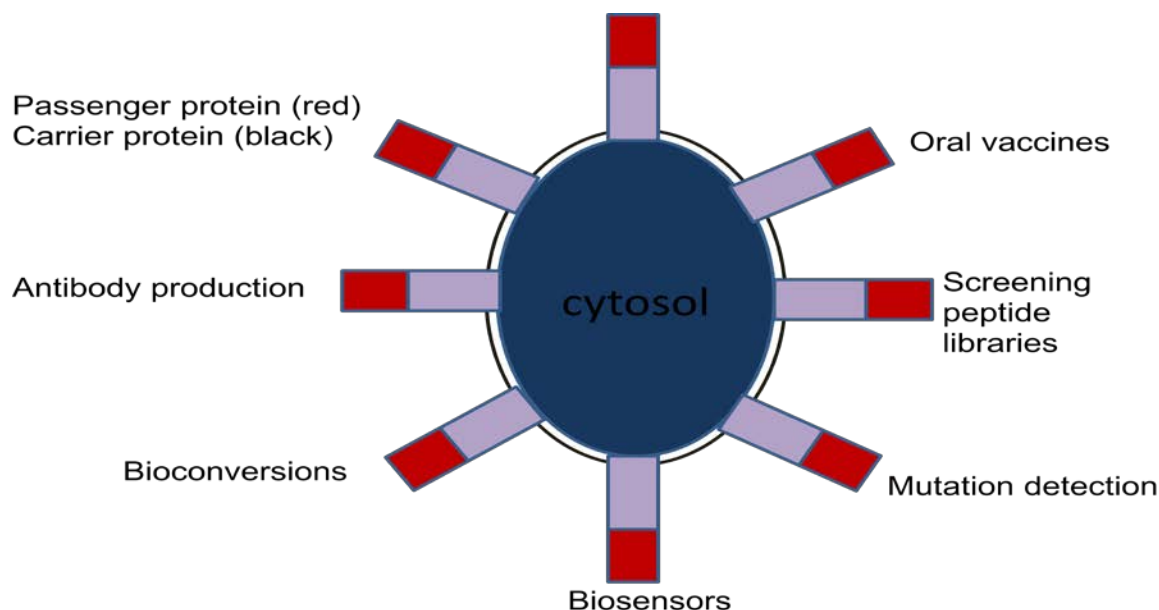


Fig.7-1.3.5. Different applications of various bio macro molecules generated in microbes

Cell-surface display allows proteins, peptides and other bio macro molecules to be displayed on the surface of microbial cells by fusing them with the anchoring motifs. The protein to be displayed -passenger protein - can be fused to an anchoring motif - the carrier protein - by N-terminal fusion, C-terminal fusion or sandwich fusion. The specific features of carrier protein, passenger protein and host cell, and various fusion methods affect the efficiency of surface display of bio-macromolecules. Microbial cell-surface display has many potential applications, including live vaccine development, peptide library screening, bioconversion using whole cell biocatalyst and bio-adsorption.

7-1.3.6 Potential applications of genetic manipulation of micro-organisms

One of the potential applications of microbial biotechnology is the production of pharmaceuticals, nutraceuticals by bacteria or other micro-organisms that produce economically, clinically important products like human insulin for diabetics or human growth hormone for dwarf individuals. Techniques are being perfected to transfer human genes into cows, sheep, and goats to obtain medically significant products from the milk of these animals.

Development of diagnostics is to detect disease-causing organisms and monitor the safety of food and water quality. Investigators are developing systems for identifying pathogens that may be used as biological weapons by rogue nations or even terrorist groups in future.

Bacteria can be genetically altered to emit a green fluorescent protein visible in ultraviolet light when they metabolize the explosive TNT leaking from land mines. Researchers envision a day when bacteria can be applied to a tract of land with a crop duster and then be analyzed from a helicopter. Genetically modified microorganisms can be used a living sensor to detect any particular chemicals in soil, air or other inorganic or biological specimens.

In Microbial Genome Program, alterations in the genome of the bacterium *Deinococcus radiodurans* are performed to increase its potential in cleaning up toxic-waste sites. The microbe's extraordinary DNA-repair processes enable it to thrive in high-radiation exposed environments.

Using various biotechnological processes, genes can be added from other organisms that will confer the ability to degrade toxinogenic chemicals such as toluene, commonly found in chemical and radiation waste sites.

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MODULE 7: LECTURE 2

ENGINEERING MICROBES FOR THE PRODUCTION OF ANTIBIOTICS AND ENZYMES

7-2.1 Introduction

Genetic manipulation of biosynthetic pathways is a useful method for producing analogs of complex bioactive metabolites. Reconstruction of biosynthetic gene clusters in *E. coli* could be done for rapid heterologous production of natural products and genetic manipulation of their biosynthetic pathways. But the real challenge lies in the suitable expression of proteins in recombinant microbe system. To obtain enzymes, antibiotics in its native, functional folded structure is the ultimate bottleneck of recombinant technology.

7-2.2 Production of Antibiotics

Antibiotics are small molecular weight compounds that inhibit or kill microorganisms at low concentrations. Antibiotics are produced by various bacteria, actinomycetes and fungi such as *Bacillus*, *Streptomyces*, and *Penicillium*. The significance of antibiotic production in micro organisms is still unclear which may be for ecological adaptation for the organism in nature.

7-2.3 Engineering *Escherichia Coli* to Produce Non- Ribosomal Peptide Antibiotics

- A monocistronic reconstituted form of the *ecm* gene (extra cellular matrix gene) cluster from *Streptomyces lasaliensis* was cloned and expressed in *E. coli* that directs the biosynthesis of the anti- tumor non- ribosomal peptide echinomycin. Biosynthetic gene function was examined by constructing a set of expression plasmids containing only 15 of the 16 genes required for echinomycin production. The *ecm18* gene cassette encoding a methyltransferase was omitted from one of the plasmids. Expression of the reconstructed cluster in *E. coli* results in production of echinomycin (red), whereas expression of the reconstructed cluster minus the *ecm18* gene results in production of triostin A (blue). It was confirmed

that Ecm18 functions as a S-adenosylmethionine dependent methyltransferases (SAM-MTs) that catalyses the conversion of triostin A to echinomycin *in vitro* using the purified recombinant enzyme.

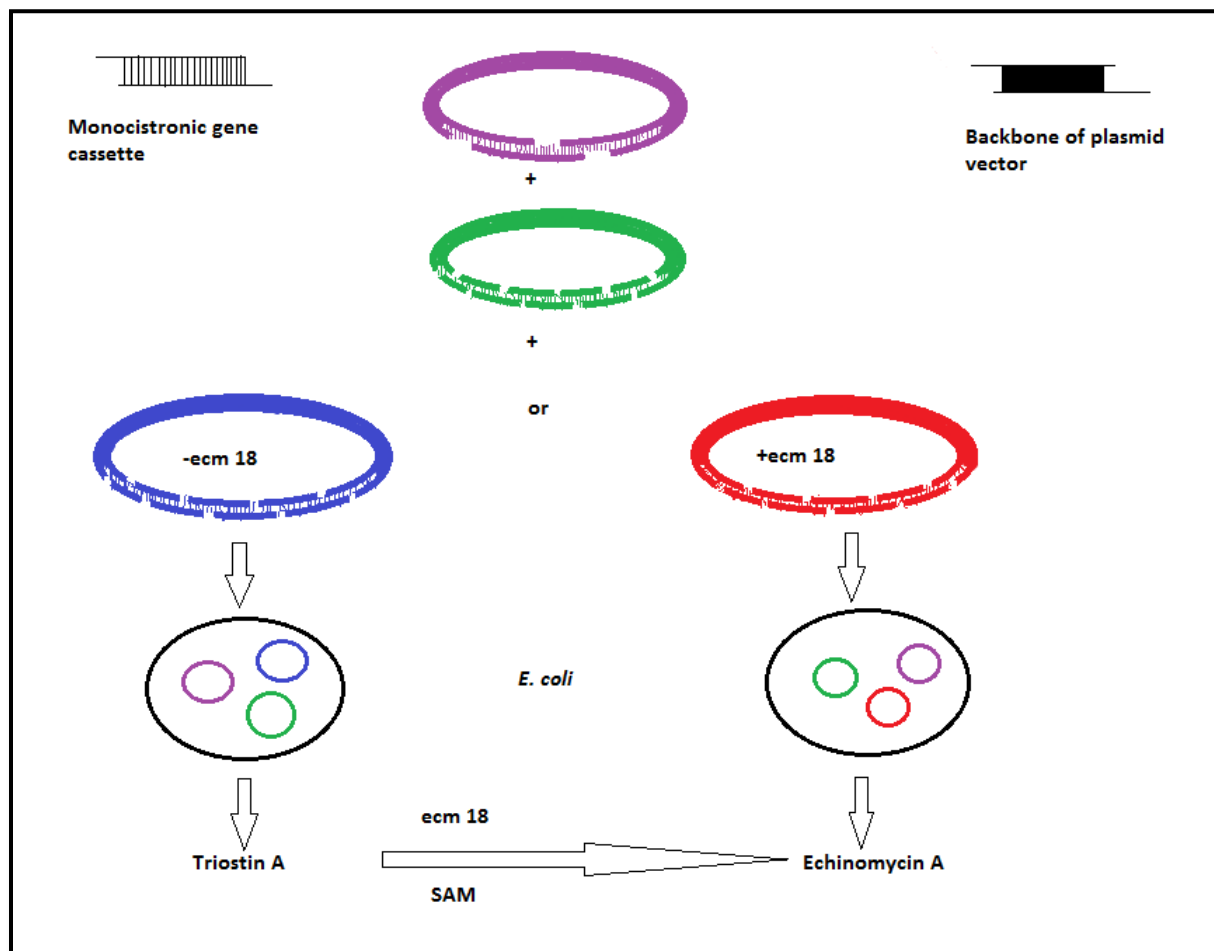


Figure 7-2.3 Strategy for reconstruction of the *ecm* cluster on three plasmids using mono cistronic gene cassettes.

Adapted from: http://www.nature.com/nchembio/journal/v2/n8/fig_tab/nchembio0806-398_F1.html

7-2.4 Metabolic Engineering in Microorganisms for Production of Antibiotics:

- Metabolic engineering considers metabolic and cellular system as an entity and accordingly allows manipulation of the system with consideration of the efficiency of the overall bioprocess, which distinguishes itself from simple genetic engineering.
- It allows defined engineering of the cell, thus avoiding unnecessary changes to the cell and allowing further engineering if necessary.

The table listed below is the compilation of different drug precursors and their host organisms and how they are genetically manipulated for the same.

Table 7-2.4 Various synthetic drug derivatives, their host and modification strategy

Drugs/drug precursors	Production host	Engineering approach
A novel amidated polyketide	<i>Streptomyces coelicolor</i>	Heterologous co-expression of amidotransferase OxyD with minimal oxytetracycline polyketide synthase in <i>S. coelicolor</i> .
Clavulanic acid	<i>Streptomyces clavuligerus</i>	Knockout of gap1 and gap2 and addition of arginine in the medium to improve the drug precursors
Daptomycin	<i>Streptomyces lividans</i>	Heterologous production of daptomycin in <i>S. lividans</i> , inactivation of actinorhodin, and optimization of the medium by adding additional phosphate
Daptomycin derivatives	<i>Streptomyces roseosporus</i>	Use of recombination to exchange single or several modules in the subunit of the non-ribosomal peptide synthase.
Erythromycin A	<i>Saccharopolyspora erythraea</i>	Overexpression of eryK and eryG with copy number ratio of 3:2
Macrolide 6-deoxyerythromycin D	<i>Escherichia coli</i>	Heterologous production of 6-deoxyerythromycin D in <i>E. coli</i> and several generations of activity-based screening assay for further evolution.

7-2.5 Production of recombinant microbial enzymes:

The production of enzymes by fermentation process was an established commercial process before modern microbial biotechnology. The microorganisms used for enzyme production are grown in fermenters using optimized growth medium. The enzymes produced by the microorganism may be intracellular or secreted into the extracellular medium.

Recombinant DNA technology was generally used for the improvement of enzyme production. Commercially important enzymes are usually of the following types:

- 1) Enzymes of industrial importance: Amylases, Proteases, Chymosin, Catalases, Isomerases recombinant Lipases.
- 2) Enzymes used for analytical purposes, such as glucose oxidase (GOs), alcohol dehydrogenase (ADH), hexokinase, cholesterol oxidases, horseradish peroxidase (HRP), alkaline phosphatase etc.
- 3) Enzymes of medicinal importance: Trypsin, Asparaginase, Proteases, Lipases etc.

Industrial enzymes have now reached an annual market of US\$1.6 billion. Recombinant therapeutic enzymes already have a market value of over US\$2 billion, being used for thromboses, gastrointestinal and rheumatic disorders, metabolic diseases and cancer. They include tissue plasminogen activator (tPA), human DNase and Cerezyme.

- The most common strategy employed to date has been the use of recombinant DNA techniques to engineer microorganisms to over-express the desired enzymes, including heterologous enzymes derived from other species which are not naturally found in the host species.
- Genetic engineering could be used to give ethanol fermenting microbes the metabolic pathways needed to utilize sugar sources such as the 5-carbon xyloses and other pentoses that are released from hydrolysis of woody biomass.

- The alternative approach would be to splice genes encoding the enzymes making up the ethanol fermentation pathway and into an organism lacking that trait but having the ability to digest the complex cellulosic components.
- A variation of this strategy is to create and use engineered microbes to manufacture novel or improved industrial enzymes, which can then be used as a catalyst in fuel fermentations to enhance or accelerate biofuel production processes. Enzymes like cellulases, amylases, and other degradative enzymes can be used to pre-treat cellulosic feedstocks, or can be added to ethanol production process at any other suitable time. Calf chymosin (prochymosin) was cloned and expressed in *E. coli* (first genetically engineered protein approved for human consumption, 1990)
- Current focus is to use synthetic biology and metabolic engineering to create novel or synthetic microorganisms possessing enzymatic capabilities not found in the original host organism.
- This strategy might involve designing an “optimal” organism using combinations of enzymes from other sources, or even completely new enzymes designed and created using protein engineering to have maximal catalytic activity.

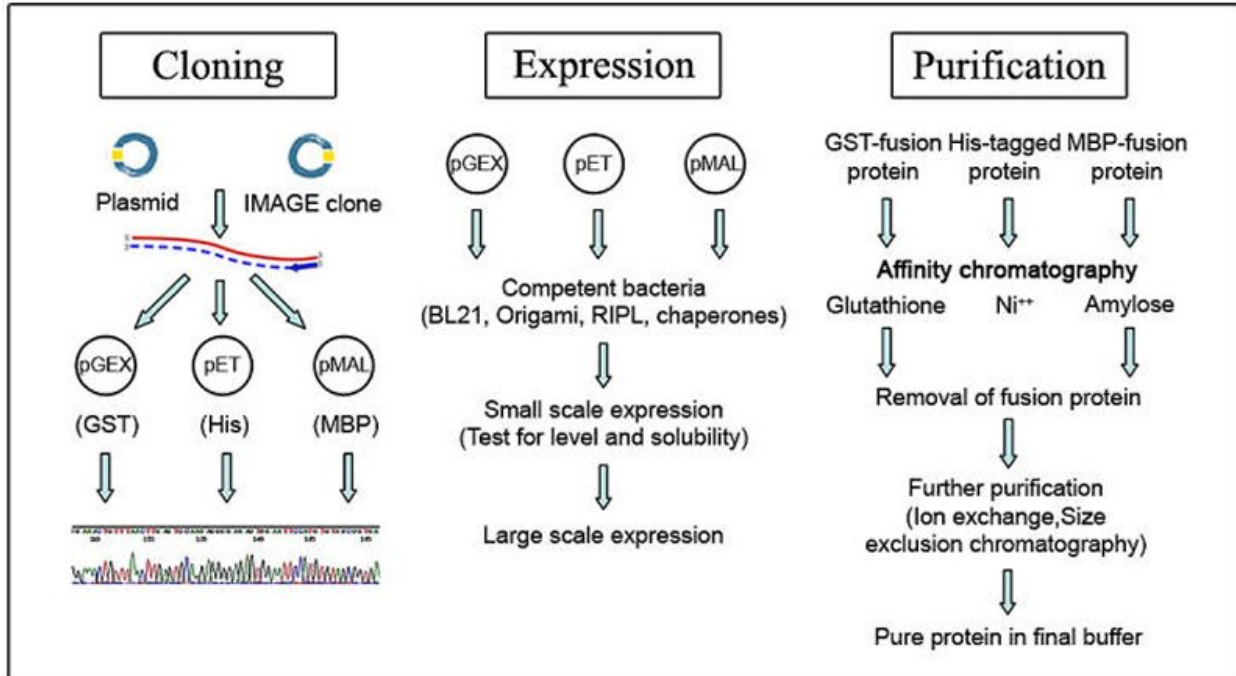


Figure7-2.5 illustrates the whole schema of recombinant protein/enzyme production in *E. coli*

(Acknowledgement: <http://www.southampton.ac.uk/cruk/protein/index.page>)

Table 7-2.5 List of industrially important enzymes produced in recombinant microorganisms:

Class	Name of enzyme	Host strains	Use of enzymes
Industrially important Enzymes	Phytase	<i>Bacillus</i> sp, <i>Aspergillus niger</i> , <i>E. coli</i>	Widely used in animal feeding to improve phosphorus nutrition and to reduce phosphorus pollution of animal waste.
	Chymosin	<i>Aspergillus niger</i> , <i>E. coli</i> , <i>K. lactis</i>	Fermentation-Produced Chymosin (FPC) used by cheese producers.
	Lipase	<i>E. coli</i>	Detergent, paper and food industry
	Pectinase	<i>Aspergillus niger</i>	Fruit juice extraction, Bioscouring of cotton fibres, textile processing, Degumming of plant fibers, Tea/coffee processing, Beer industry
	Alpha-Amylases	<i>Bacillus licheniformis</i> <i>Bacillus stearothermophilus</i> , <i>Bacillus amyloliquefaciens</i>	Fungal amylases (<i>Aspergillus</i> sp. <i>Penicillium</i> sp.) Various applications in Paper industry, food industry, textile industry, detergent industry.
Enzymes used for analytical purposes	Glucose oxidases	<i>Aspergillus niger</i>	Exploited to develop biosensor in nano-diagnostics,
	alcohol dehydrogenase (ADH)	<i>Lactobacillus brevis</i>	In fuel cells, alcohol dehydrogenases can be used to catalyze the breakdown of fuel for an ethanol fuel cell
	cholesterol oxidases	<i>E. coli</i>	To control cholesterol level inside the cell
	horseradish peroxidase (HRP)	<i>E. coli</i>	Often used as a conjugate to detect the target antigen or other bio-molecules
	Alkaline phosphatases	<i>E. coli</i>	hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins or alkaloids
Enzymes of medicinal importance	Trypsin	<i>E. coli</i>	Helps in cleavage of proinsulin to insulin and
	Asparaginase	<i>Erwinia aroideae</i> NR RL B-138	Causes the hydrolysis of asparagine to aspartic acid, Also used as a drug “Elspar” for the treatment of

			acute lymphoblastic leukemia (ALL)
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7-2.6 Genetic engineering in *Methylophilus methylotrophus*:

Methylophilus methylotrophus, the obligate methylotrophs are able to efficiently convert methanol to single-cell protein, a process of major importance to a variety of industries.

The glutamate dehydrogenase gene of *E. coli* has been cloned into broad host range plasmid and can complement glutamate synthase mutants of *Methylophilus methylotrophus*. Assimilation of ammonia via glutamate dehydrogenase is more energy efficient than via glutamate synthase. Thus the recombinant microorganism can convert more growth-substrate, methanol into cellular carbon.

Trans-conjugants were selected for the antibiotic resistance encoded by the vector, and the GDH enzyme activity measured to confirm the presence of the GDH gene.

The strain constructed by these manipulations was able to convert methanol to single-cell protein more efficiently than the original parent strain.

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MODULE 7- LECTURE 3

ENGINEERING MICROBES FOR THE PRODUCTION OF INSULIN, GROWTH HORMONES AND MONOCLONAL ANTIBODIES

7-3.1 Introduction

Gene manipulation technology is the most important tool considered as the back bone of modern biotechnology. Presently diverse techniques are involved in the production of insulin, growth hormone and monoclonal antibodies. These are the modern medicines produced by the genetically engineered organisms (FDA approved GRAS –generally regarded as safe organisms). Production of human insulin by recombinant *E. coli* is considered as a significant outcome of recombinant DNA technology, more complex proteins of medical uses can also be produced by metabolic and cellular engineering of microorganisms. But production of proteins and other derivatives in its native, functional and intrinsic condition is the ultimate challenge of recombinant technology.

7-3.2 Production of Insulin:

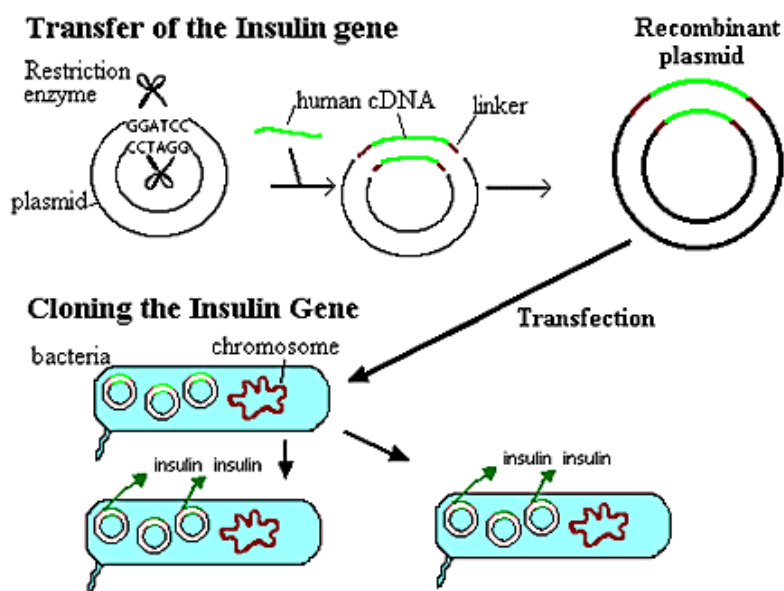
Insulin is a peptide hormone mainly used in treatment of diabetes mellitus to control elevated blood glucose level. Banting and Best named it originally as 'isletin' and was later renamed as insulin by Macleod, a word that had been suggested in 1910. This hormone is secreted by the β -cells of the pancreas and consists of two polypeptide chains, A and B which are linked by two inter-chain and one intra-chain disulphide bridge. Insulin is synthesized as a single-chain precursor, pro-insulin, and produced by the proteolytic processing of pro-insulin in the pancreas (Kjeldsen *et al* 1999).

Originally insulin was first identified from dog pancreas which was commercially produced from various sources like foetal calf pancreas obtained from slaughter houses. Now human insulin protein is mass-produced through genetic engineering processes. Recombinant DNA technology has been a great enabler in producing human insulin outside the body for being used as a therapeutic. Insulin is the first human hormone produced in bacteria to be tested in humans for medical purposes.

There are many methods for the production of recombinant human insulin in both bacteria and yeast. One typical scheme for preparing human insulin utilizes pro-insulin that is produced in *E. coli* cytoplasm as an inclusion body of a fusion protein (Chang 1998).

Manufacturing of insulin using microbes as a cell factory involves the following steps –

1. Isolation of gene: The gene for producing human insulin protein is isolated.
2. Preparation of target DNA: Circular piece of DNA called plasmid is obtained from bacteria.
3. Insertion of DNA into plasmid: The gene for insulin is inserted into the plasmid construct. The human insulin gene is now recombined with bacterial DNA.
4. Plasmid insertion: The bacterial DNA having insulin gene is inserted back into bacteria.
5. Plasmid multiplication: The bacterial cells having insulin gene are allowed to grow and multiply and during this process bacterial cells start to produce recombinant insulin. During division newly synthesized copy of cell are produced.
6. Human insulin produced by bacteria is purified.



Transfer and cloning of the Insulin gene

Figure 7-3.2 Production of insulin by genetic engineering

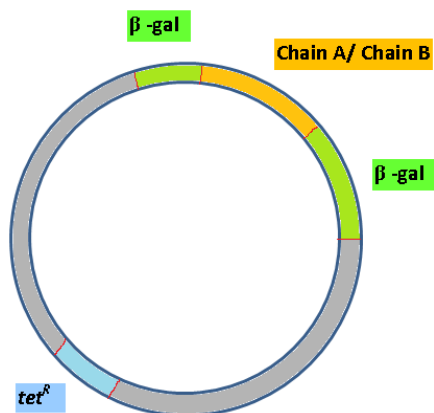


Fig7-3.2.1 Map of the recombinant plasmid vector containing cDNA sequence for insulin polypeptide (Chain A or B)

7-3.3 Production of Growth Hormones:

Growth hormone is one of the most important hormones in human body. The core center for production of growth hormone is pituitary gland. The action of growth hormone is either direct or indirect on the human physiological process. But in some children, malfunction of growth hormone results in abnormal growth of the individual. In case of these conditions recombinant growth hormone is useful for the treatment.

Human growth hormone has versatile functions:

- Activates the production of protein in cells by releasing some essential factors.
- Helps in fastening the production of DNA and RNA.
- Accelerates the generation of red blood cells and augments the flow of blood to the kidneys and the rate at which the kidney does its vital filtration work.
- Plays a major role in maintaining the level of fats in the body.
- Activates bone growth and skeletal development indirectly by producing intermediate factor IGF-1.

Table 7-3.3 Metabolic engineering of microorganisms to exploit the hormones:

Hormone	Production host	Engineering approach
Gonadotropin-releasing hormone	<i>Escherichia coli</i>	Heterologous expression of the recombinant gonadotropin-releasing hormone in <i>E. coli</i> using a T7 RNA polymerase-based expression system and evaluation of various culture conditions on the plasmid stability and the product yield.
Human growth hormone	<i>Escherichia coli</i>	Activation of the promoter lambda PL by temperature shift for production of human growth hormone without contaminants.
Human parathyroid hormone	<i>Escherichia coli</i>	Using recombinant <i>E. coli</i> strain BL21 (DE3) harboring the plasmid. pET32aBI1 encoding the fusion gene of thioredoxin and human parathyroid hormone

In order to provide large quantities of IGF-I for physiological investigation and clinical trial,

rDNA technology has become the method of choice since large amount of exogenous proteins could be expressed in bacteria. The prokaryote *E. coli* is preferred as host because of its ease of handling and cultivation, and high yields for many recombinant proteins. Several literature references exist on the production of IGF-I in bacteria as a secreted form fused to secretion leader sequences. (Kim *et al* 1996)

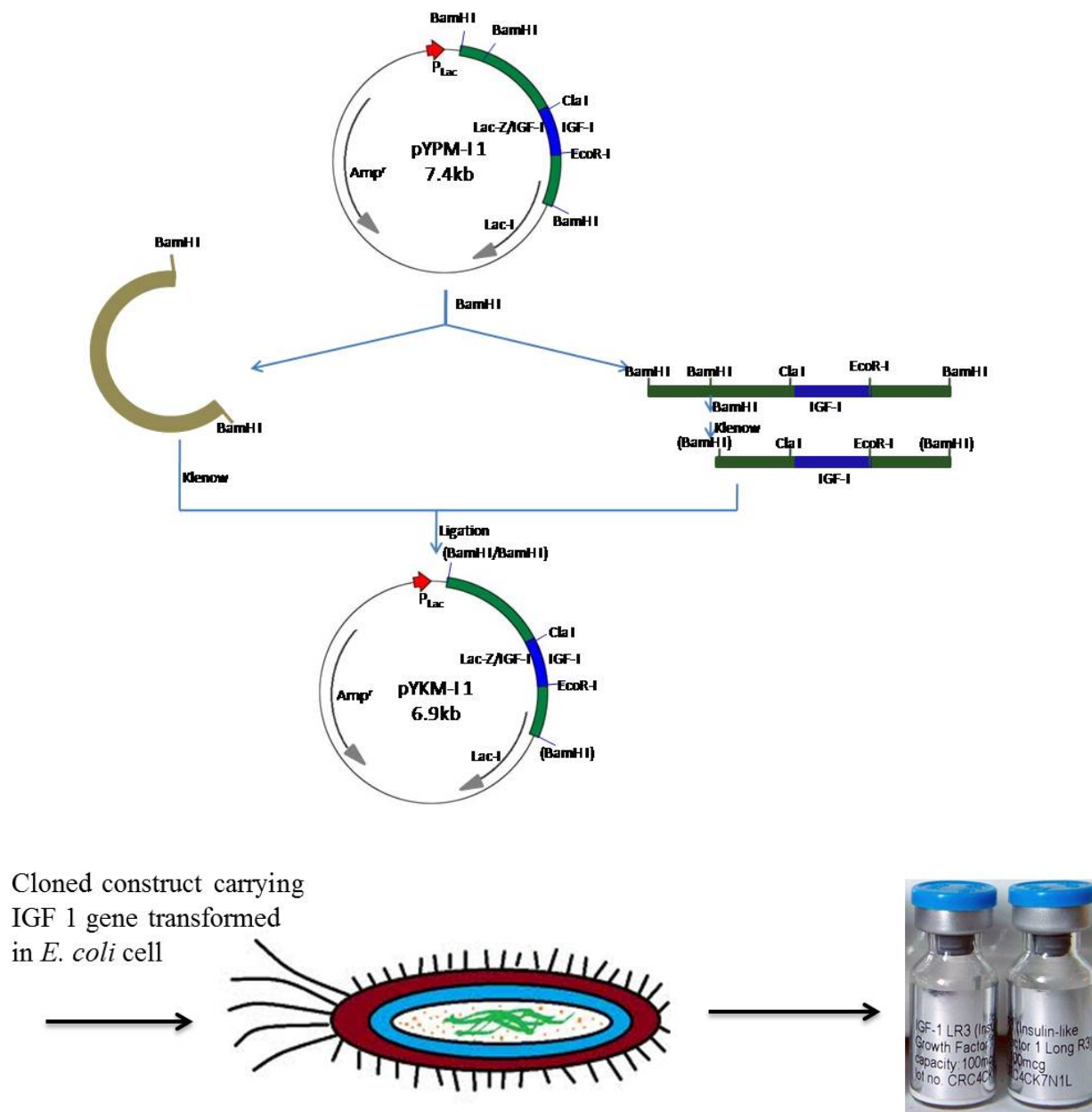


Fig 7-3.3.1. Outline scheme for production of recombinant IGF-I.

(Ref. Kim et al. Journal of Biotechnology 48 (1996) 97-105)

Lately, biologically and highly active recombinant human insulin-like growth factor-I (rhIGF-I) was produced in yeast (*S. cerevisiae*). rhIGF-I is a 7.5kDa protein containing 70 amino acid residues, which stimulates the proliferation of a wide range of cell types including muscle, bone and cartilage tissue. IGFs control the biosynthesis of many intracellular and extracellular components and are potent mitogens for MDCs (mesenchymally derived cells). Growth hormone has been shown to mediate its effects on bone formation indirectly, through IGF-I. rhIGF-I is also known as Somatomedin-C is effective and its use apparently involved no special hazards.

Recombinant DNA technology is mainly used as a key for production of the growth hormone. In 1979, Dr. Baxter's team and a group of scientists at Genentech succeeded in producing human growth hormone in genetically modified bacteria. Recently, these artificially derived biosynthetic growth hormones are widely exploited in humans.

7-3.4 Production of Monoclonal Antibodies (mAb) Using Microorganisms as Cell Factories:

Monoclonal antibodies are specific antibodies which bind to the particular site of proteins i.e. epitope. Production of the monoclonal antibodies was done from the identical immune cells. They had a major role in treatment of cancer due to their site specificity.

Major techniques involved in the production of recombinant monoclonal antibodies were repertoire cloning or phage display/yeast display. Recombinant antibody techniques use viruses and yeast as a cell factories for the production of monoclonal antibodies.

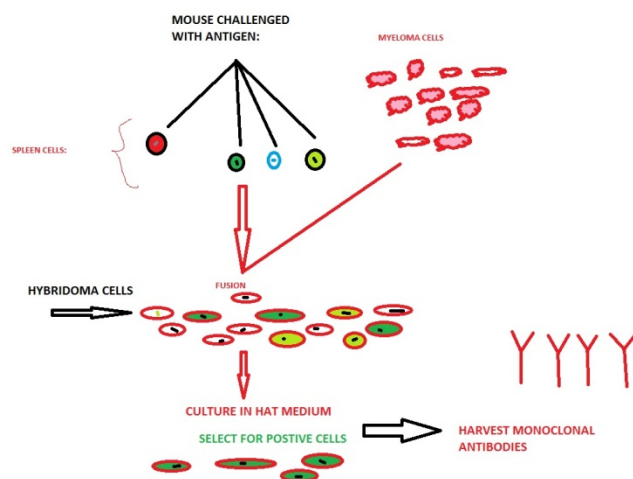


Figure7-3.4 Conventional protocol for production of monoclonal antibodies

- 1) Phage antibody libraries are a variant of the phage antigen libraries first invented by George Pieczenik.
- 2) All the recombinant antibody techniques rely on cloning the specific gene segments to create the libraries of antibodies. These libraries are specifically different in only few amino acids which decide their specificity.
- 3) These techniques are applied to improve the specificity with which antibodies recognize antigens, their stability in various environmental conditions, their therapeutic efficacy, and their detectability in diagnostic applications.

Monoclonal antibodies production from the fungal species is preferred over the *E. coli* as:

- a) Antibodies produced from the fungal systems are extracellular which make protein separation process easier. However recovery of antibodies from the *E. coli* is very intricate due to its periplasm.
- b) Common fungal organism exploited for the production of monoclonal antibodies with single chain is the *Aspergillus niger*.
- c) Ward *et al.* expressed full-length IgGs in *A. niger* using an N-terminal fusion to glucoamylase for both light and heavy chains. They relied on the endogenous KexB protease in *A. niger* to cleave off the fusion during the secretion of the antibody. (Ward M *et al*, 2004)
- d) Monomeric scFv production in *P. pastoris* was also demonstrated and optimized. (Cunha *et al*, 2004)

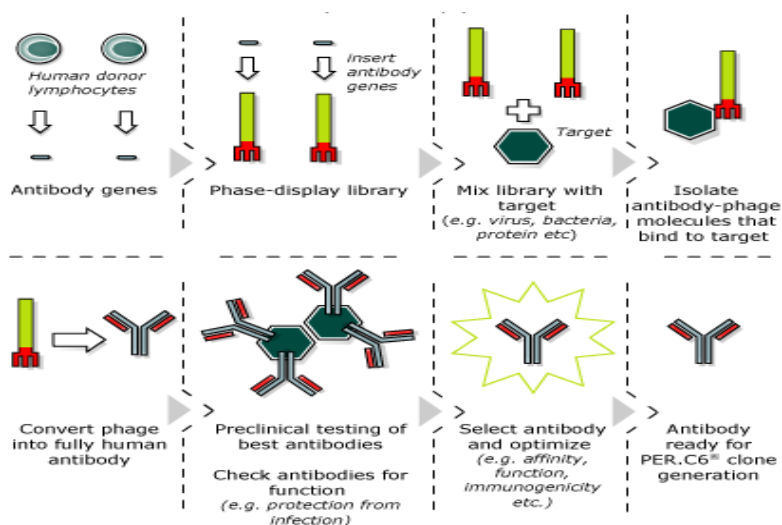


Figure7-3.4.1 Flow diagram of human mAb discovery process

(Acknowledgement: <http://www.crucell.com/>)

Improvements in the microbial production of antibodies and fragments have resulted from host-cell engineering to give increased and optimized productivity. There is also a trend of producing antibodies or fragments with increased circulating half-life.

7-3.4.1 Applications of recombinant Monoclonal Antibodies:

a) Diagnostic Applications: Monoclonal antibodies can be used as a specific probe for developing biosensors and microarray systems.

b) Therapeutic Applications:

- Transplant rejection can be detected immediately with the MAB and CD marker conjugates.
- Most common monoclonal antibodies like Abciximab, Cetuximab are widely prescribed for the cardiovascular diseases and cancer respectively. For breast cancer treatment MAB like Herceptin is a breakthrough invention.
- For treatment of infectious diseases Palivizumab and Briakinumab are widely used whereas for the inflammatory diseases Infliximab are commonly used.

c) Future applications: By exploiting monoclonal antibodies we can combat against the terrorists who can cause threat using biological organisms (Bioterrorism).

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MODULE 7- LECTURE 4

ENGINEERING MICROBES FOR CLEARING OIL SPILLS

7-4.1 Introduction

An oil spill is the release of a liquid petroleum hydrocarbon into the off shore and on shore environment due to human activity and is a form of serious pollution. Oil spills occur due to releases of crude oil from tankers, offshore platforms, drilling rigs and wells, as well as spills of refined petroleum products (such as gasoline, diesel) and their by-products, heavier fuels used by large ships such as bunker fuel, or the spill of any oily refuse or waste oil. Another significant route by which oil enters the marine environment is through natural oil seeps. Natural oil seeps generally occur with slow rate while due to human activity are sudden, high in volume and are thus catastrophic. Some of the effects of oil spills are discussed below:

7-4.2 Environmental effects of Oil Spill

- Oil impairs a bird's ability to fly, preventing it from foraging or escaping from predators. Birds may ingest the oil which causes irritation in the digestive tract, altering liver function, and causing kidney damage.
- Oil penetrates into the structure of the plumage of birds and thus reduces its insulating ability and making them more vulnerable to temperature fluctuations and much less buoyant in the water. It can cause dehydration and metabolic imbalance in birds.
- Some birds also experience changes in their hormonal balance including changes in their luteinizing protein.
- Furred marine mammals exposed to oil spills are affected in similar ways as birds.
- Animals can be poisoned, and may die from oil entering the lungs or liver.
- It leads to interrupt the food chain on which fish and sea creatures depend, and on which their reproductive success is based.

7-4.3 Cleanup and Recovery:

Cleanup and recovery from an oil spill is difficult task. Although the physical removal of spilled oil, cleaning and decontaminating the area assists large-scale recovery of the environment, but may harm the substrate biomass.

Cleanup and recovery depends upon following factors some of which are interrelated;

- type of oil spilled,
- water temperature (affecting evaporation and biodegradation),
- types of shorelines and beaches involved
- ecological protection,
- socioeconomic effects,
- health risk

7-4.3.1 Environmental Recovery Rates:

The rate of recovery of the environment after an oil spill occurs depends on

- oil composition
- properties and the characteristics of the area impacted,
- the outcome of intervention and remediation measures.

7-4.3.2 Methods for Cleaning Oil Spills:

Cleaning up oil spills involve an array of physical, chemical and biological methods as discussed below;

1. **Booms**–Booms are temporary floating barriers used to contain an oil spill. They help in concentrating that so skimmers, vacuum can easily collect or any other collection method.
2. **Skimming**- Skimming is based on the principle that oil being lighter than water floats on its surface and thus can be easily collected and removed. Skimming system involves boat with boom that collects oil, a boat with large tank that hold oil and an actual sucker.

Skimming depends on thickness of the oil slicks, the amount of debris mixed with oil, the consistency of the oil and current state of sea.

3. Shovels and other road maintenance equipments are used to clean up oil on beaches.

4. Solidifying- Solidifiers are composed of dry hydrophobic polymers that adsorb oil. Solidifiers are insoluble in water; change the physical state of spilled oil from liquid to a semi-solid or a rubber-like material that floats on water making the removal of the solidified oil easy. The time required for solidification of oil is controlled by the surface area or size of the polymer as well as the viscosity of the oil.

Advantages of solidifying:

- Non-toxic to aquatic and wild life.

Suppresses harmful vapors commonly associated with hydrocarbons such as Benzene, Xylene, Methyl ethyl, Acetone and Naphtha

5. Chemical dispersants - Chemical dispersants are materials that break down the oil into simpler chemical constituents and help to disperse the oil to make it less harmful to wildlife and shorelines.

6. In-situ burning- This involves the burning of freshly spilled oil while it's still floating on the water under favorable conditions of low wind. However this causes air and water pollution and harmful to aquatic life.

7. Biological entities like microorganisms (microbial bioremediation) or plants (phytoremediation) or biological agents are used to break down or remove oil.

Anaerobic Sulfate-reducing bacteria (SRB), acid-producing bacteria and aerobic-general aerobic bacteria (GAB) are naturally oil-consuming bacteria. These bacteria occur naturally and act to remove oil from an ecosystem. In an oil spill their biomass will tend to replace other populations in the food chain.

It is worth remembering that in nature there is no single strain of bacteria having metabolic capacity to degrade all the components found within crude oil. Biodegradation of crude oil involves a succession of species within the consortia of microbes present.

To get better results, cleaning of oil spills is often deployed as an integrated operation of physical, chemical and biological means. For e.g. The slick is barricaded by physical structures and chemical dispersants are applied within the barricade both above and below the surface of the oil slick to break the oil into smaller droplets making it easier for bacteria to degrade it.

7-4.3.3 Costs and Prevention

Prevention of oil spills is given a major priority due to its negative environmental-socio-economic impact. However, the high costs associated with oil spills and regulations governing offshore facilities and operations have necessitated the development of improved technology for efficient spill prevention. The costs of an oil spill can be viewed in both quantitative and qualitative terms of references.

Quantitative costs of an oil spill include the following,

- loss of the oil,
- payment for cleaning up the spill and remediating the environment,
- repair of physical facilities,
- penalties assessed by regulatory agencies,
- expenses in insurance and legal claims.

Qualitative costs of an oil spill include following,

- the loss of pristine habitat,
- biodiversity both known and unknown
- human health effects from exposure to water and soil pollution.

Estimation of the volume of a spill

An oil slick in the open ocean is usually a very thin layer of oil covering a large area. The volume of an oil spill is computed as below;

$$V_{os} = A_{os} \times D_{os}$$

Where, V_{os} = Volume of the oil spill,

A_{os} = Area covered of the oil spill,

D_{os} = Average thickness of the oil spill.

A_{os} is measured with satellite imagery and is a fairly a simple and accurate process. Using the image a line around the visible edges of the slick is traced and the area inside the boundary is computed. For oil spill reports where there is no imagery, the reported length and width of the slick to compute the rectangular area containing the slick is used.

D_{os} is estimated by observing the color of an oil spill. The thickness is assigned based on established guidelines for the range of thicknesses that can produce a slick of that color (e.g. "Rainbow sheen"). .

7-4.4 Microbial bioremediation and GEMs

Microbial bioremediation is the process in which microorganisms like bacteria degrade or transform hazardous organic compounds like benzene, toluene, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), dioxins, nitro-aromatics etc. into non-toxic substances.

Naturally occurring microorganisms are incapable of degrading all toxic chemicals, especially xenobiotics. To overcome this, attempts have been made in recent years to create genetically engineered microorganisms (GEMs) to enhance bioremediation beside degrading xenobiotics. In spite of this the number of field trials for the use of genetically engineered microorganisms for bioremediation still remains limited (Peiper *et al* 2000, Sayler *et al.* 2000). The history of genetic engineering is strongly linked with the bioremediation of oil spills.

In 1971, the great scientist Prof. A M Chakrabarty had found four different strains of the common *Pseudomonas* bacteria that contained enzymes which can break down various hydrocarbons. He also observed that the genes for oil-degrading enzymes were located on the extra-chromosomal elements known as plasmids. By combining these plasmids into a strain of *Pseudomonas*, he created a variant of *Pseudomonas* that was capable of breaking down the constituents of crude oil. The plasmids of *Pseudomonas putida* degrading various chemical compounds are TOL (for toluene and xylene), RA500 (for 3, 5-xylene) pAC 25 (for 3-chlorobenzoate) and pKF439 (for salicylate toluene). Plasmid WWO of *Pseudomonas putida* is one member of a set of plasmids now termed as TOL plasmid.

These new superbug is claimed to have the potential to degrade oil 10–100 times faster than other non-genetically engineered independent strains.

However due to regulations and concerns of the public using the microbe for bioremediation, the strain was never unused.

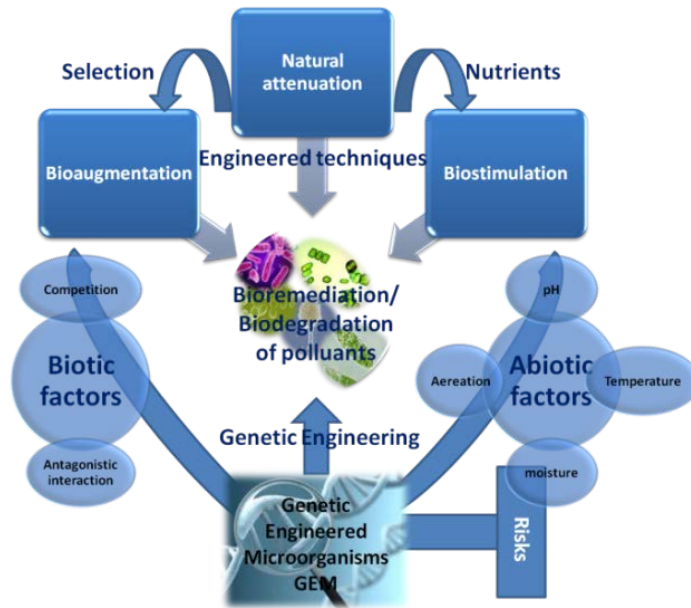


Fig 7-4.4. Diagrammatic representation of biodegradation involving microorganisms and GEM

(Obtained from:<http://www.intechopen.com/books/biodegradation-life-of-science/biodegradation-involved-microorganisms-and-genetically-engineered-microorganisms>)

Table 7-4.4.1 List of organisms degrading various petroleum hydrocarbons

Organism	Degrading Hydrocarbon(s)
<i>Azoarcus sp.strain EBI</i>	Ethyl benzene
<i>Azoarcus sp.strain T</i>	Toluene, m-Xylene
<i>Azoarcu stolulyticus</i>	Toluene, m-Xylene
<i>Pseudomonas sp.NAP3,EbN1,HdN1,M3,T3, ToN1</i> <i>Vibrio sp. Strain NP4</i>	Napthalene
<i>Thauera aromatica K172,ThaueraaromaticaT1,</i> <i>Geobacter grbiciae TACP5,Desulfobacterium</i> <i>cetonicum</i>	Toluene
<i>Desulfobacterium cetonicum strain AK-O1</i>	C13-C18 alkanes
<i>Desulfobacterium cetonicum strain NaphS2</i>	Napthalene
<i>Desulfobacterium cetonicum strain TD3</i>	C6-C16 alkanes

Compiled from Bernard Ollivier, Mitchel Magot, Petroleum Microbiology, Amer Society for Microbiology, 2005

Approaches to GEM development for bioremediation application (*Menn et al 2008*)

- 1) Modification of enzyme specificity and affinity;
- 2) Pathway construction and regulation;
- 3) Bioprocess development, monitoring and control;
- 4) Bioaffinity/ bioreporter sensor applications for chemical sensing, toxicity reduction and end point analysis.

Genes responsible for degradation of environmental pollutants, for example, toluene, chlorobenzeneacids, xylene and other toxic wastes have been identified. For every compound, one separate plasmid is required. One single plasmid cannot able to degrade all the toxic compounds of different groups.

The plasmids are grouped into four categories:

- 1) OCT plasmid which degrades, octane, hexane and decane;
- 2) XYL plasmid which degrades xylene and toluenes,
- 3) CAM plasmid that decompose camphor and
- 4) NAH plasmid which degrades naphthalene (Ramos JL *et al*).

The potential for creating microbial strains through genetic manipulation, which has ability to degrade a variety of hydrocarbons, has been demonstrated by Markandey *et al*. They successfully developed a multi plasmid-containing *Pseudomonas* strain capable of oxidizing aliphatic, aromatic, terpenic and polyaromatic hydrocarbons. GEM like *Pseudomonas putida* that contained the XYL and NAH plasmid as well as a hybrid plasmid derived by recombination of CAM and OCT developed by conjugation could degrade camphor, octane, salicylate, and naphthalene and could grow rapidly on crude oil because its capabilities of metabolizing hydrocarbons more efficiently than any other single plasmid.

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